

PRODUCTION OF CIGUATOXINS IN CULTURED *GAMBIERDISCUS TOXICUS*

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Production of ciguatoxin congeners (CTX) from mass cultured dinoflagellates appears to be the only source of CTX that has the potential of providing sufficient quantities of purified toxins for studies on biosynthesis, structural analysis, pharmacology, biotransformation and detection. Established *Gambierdiscus toxicus* clones and recent isolates from Tahiti, Guam and Grand Cayman Island were mass cultured and toxins separated by step-wise elution on a silica gel column. CTX was identified by its binding competition with [³H]-brevetoxin for sodium channel receptor sites in rat synaptosomes. MTX was identified by its ability to induce calcium flux activity in rat pituitary cells. Although the silica gel column separated CTX from MTX, general toxicity of the CTX congeners decreased after separation. A sample of partially purified CTX from *G. toxicus* clone MQ2 was used as a standard for evaluating the assays.

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Two classes of cyclic polyethers produced by the epiphytic dinoflagellate, *Gambierdiscus toxicus*, are non-polar ciguatoxins (CTX), the principal toxins causing ciguatera, and polar maitotoxins (MTX). CTX was first isolated from the Pacific red snapper (Scheuer et al., 1967) and later purified from moray eel liver and viscera. Multiple congeners of CTX (CTX-1, CTX-2 and CTX-3) have been identified in finfish (Lewis et al., 1991), CTX-1 being identical to CTX from moray eel liver. Several CTX congeners have been identified in wild and cultured *G. toxicus* (Legrand et al., 1992; Holmes et al., 1991; Satake et al., 1993) although none identical to that found in the moray eel. Based on their structural relationships, Lewis et al. (1991) proposed that CTX-1 and CTX-2 in finfish represent oxidation products of two different toxins in *G. toxicus*.

Even in the moray eel, which is the most toxic species, CTX content is extremely low, usually only several ppb in whole bodies (Murata et al., 1990). Thus purification of large quantities of CTX from finfish has not been successful. However, obtaining large quantities of the dinoflagellate CTX congeners is limited only by the ability to identify and mass culture a *G. toxicus* clone capable of producing high levels of these toxins. While MTX levels greatly exceed those of CTX, strain-dependent differences in the composition of toxins produced by cultured *G. toxicus* have been described (Holmes et al., 1991). Toxin profiles appeared to be stable, suggesting a

genetic basis for the production of different toxin profiles. However, no isolate has yet produced substantial quantities of CTX in culture, with reported yields less than one mouse unit per million cells (Holmes et al., 1991; Satake et al., 1993).

We proposed to screen a collection of 44 *G. toxicus* clones from 12 geographically distinct locations worldwide for their production of CTX in mass culture. The screening of clones has traditionally been a laborious task, requiring the use of mouse bioassay to identify toxins. Since CTX and MTX elicit similar symptoms in the mouse bioassay, the identification of CTX versus MTX in dinoflagellate extracts has required extensive purification. Our laboratory is capable of distinguishing and quantifying CTX and MTX in crude extracts of *G. toxicus* using a battery of established bioassays. These include a rapid, high-throughput *in vitro* cytotoxicity assay for total toxicity, and a Ca²⁺ flux assay as well as receptor binding competition between CTX and [³H]-brevetoxin to distinguish MTX from CTX (Van Dolah et al., in press). Maitotoxin causes an increase in Ca²⁺ permeability, possibly through voltage dependent Ca²⁺ channels (Takahashi et al., 1982; Gusovsky & Daly, 1990). Ciguatoxin promotes Na⁺ channel opening by binding to site 5 on the voltage dependent sodium channel (Lewis & Endean, 1984; Bidard et al., 1984). This site is also recognized by the brevetoxins, a related class of dinoflagellate polyether toxins that can be displaced by CTX (Lombet et al., 1987;

Baden, 1989). The objective of this study was to develop a rapid, single column technique to separate CTX from MTX in crude dinoflagellate extracts. This would expedite identifying *G. toxicus* clones which produce high levels of CTX congeners in mass culture.

MATERIALS AND METHODS

STOCK CULTURES

Isolation procedure for clonal cultures followed Babinchak et al. (1986). Stock cultures of clonal strains of *Gambierdiscus toxicus* (Table 1) were maintained at 27°C under an illumination of 30–40 $\mu\text{EM}^{-2}\text{S}^{-1}$ and a 16:8 hour light:dark cycle without aeration. Illumination was provided from above by a 50:50 mixture of Cool White (North American Phillips Lighting Corp.) and Vita-Lite (Duro-Test Corp.) fluorescent bulbs. K medium (Keller et al., 1987), an enriched seawater medium used in all culturing, was modified by eliminating CuSO_4 , Tris buffer, silica and using ES vitamin concentrations (Guillard & Keller, 1984). Seawater was collected from a saltwater well in Vero Beach, FL, (Florida Institute of Technology field station). The seawater (35–36 ‰) was filtered through 0.45 μm cartridge filters into sanitized polycarbonate carboys and refrigerated in the dark. The seawater was autoclaved in 10 litre borosilicate glass bottles. The vitamin mixtures and enrichments for K medium were prepared in concentrated stocks, filter-sterilized and autoclaved respectively, and stored frozen. The enrichment and vitamins were added aseptically to autoclaved seawater which was then used immediately for culturing. *G. toxicus* clones were harvested by filtration through 12 μm polycarbonate membranes, washed 3 times with sterile seawater and inoculated at 200–300 cells/ml into 2.8 litre Fernbach flasks containing 1 litre of medium. Stock cultures of *G. toxicus* were harvested for transfers and mass culture inoculum at 10–14 days.

MASS CULTURE

Twenty-nine clones (Table 1) were selected for mass culture. Micro-carrier spinner flasks (Bellco Glass, Inc.), designed for suspension cell culture systems, were selected as mass culture vessels. The 8 litre model was of a design, wide mouth with two access ports, and weight that could be easily handled and cleaned. The maximal working volume of these flasks for dinoflagellate culture was increased to 12 litre. Magnetic stirring units (Bellco Glass, Inc.), designed for gentle

agitation, maintained a stirring speed of 20 RPM. Shelving units accommodated 6 culture vessels and stirrers and provided illumination from above with 4ft Vita-Lite fluorescent bulbs, and from behind with 2ft Cool White fluorescent bulbs at 40 $\mu\text{EM}^{-2}\text{S}^{-1}$ using a 16:8 hour light:dark cycle. Two shelving units were installed in each of two walk-in environmental rooms (1800 cu. ft. and 600 cu. ft.). These rooms were maintained at 27°C and provided space for 24 culture vessels. Vessels were inoculated at a concentration of 500–1000 washed cells/ml. Air was supplied at 1 litre min^{-1} by Whisper 800 aquarium air pumps (Willinger Bros., Inc.) and filtered through AQ microfiber disposable filter tubes, (Balston Filter Products). Aeration was initiated after 10–14 days incubation and bubbled into the culture vessels through sterile plastic air diffusers, (Lee's Aquarium Products).

HARVESTING PROCEDURE

Harvesting the micro-carrier spinner flasks required minimal handling of the vessels. After 21–28 days of incubation, the stirring and aeration apparatus were removed, the flask swirled and a 10ml sample taken for determining cell counts. The cells were allowed to settle and the supernatant removed with a peristaltic pump and filtered through a 142mm stainless filter holder (43 μm polyester membrane, Spectrum Medical Industries, Inc.). The settled cells from all the vessels were combined and collected on a 43 μm polyester membrane in a 90mm glass filter holder which produced a cake of wet cells. The cells were stored frozen at -20 or -90°C until extracted for toxin content.

CELL COUNTS

Cell counts on individual micro-carrier flasks were determined at harvest using natural chlorophyll autofluorescence and direct epifluorescence microscopy. Duplicate 0.5ml volumes of each micro-carrier sample were collected on 5 μm black polycarbonate membranes and observed at 320x on a video display monitor. A Leitz Dialux 20 microscope was used, equipped with a 150W xenon lamp, fluorescence vertical illuminator, KG1 heat filter, BG23 blue filter and a Leitz I2 filter block. Twelve fields or a minimum of 400 cells per sample were counted.

PREPARATION OF CTX STANDARD

A 150g (wet weight) sample of clone MQ2 was disrupted in 100% methanol at 0°C with a Tekmar 500 watt sonic disrupter and filtered through a

Table 1. *Gambierdiscus toxicus* Culture Collection†, National Marine Fisheries Service, Charleston Laboratory

CLONE	ORIGIN	ISOLATOR
*CI03, *CI04, *CI05, CI08, CI09, CI10, CI12, CI13, CI14, CI15, *CI16, *CI18	Grand Cayman Is.	Babinchak
CZ2, CZ3, CZ4	Cozumel, Mexico	Babinchak
MQ1, *MQ2	Martinique	Babinchak
G03, *G05, #G06, *G15, *G16, *G17	Guam	Babinchak
#G02,	Palau	Babinchak
*G01, G20, *G23	Pohnpel, FSM	Babinchak
*MR-I	Moorea	Babinchak
*T01B, *T02B, #T03B, *T04B, *T11B, *T15B	Tahiti	Babinchak
*T01,	Tahiti	Bagnis
*AUS-1	Australia	Bomber
SB01, SB03, SB04	St. Barthelemy	Durand-Clement
*T04	Tahiti	Durand-Clement
*HIT-10, *HIT-25	Tahiti	Legrand
#TP125B	Dry Tortugas	Tomas
*T39	Hawaii	Withers

* Clones successfully mass cultured in micro-carrier system

Clones not successful in mass culture

†*G. toxicus* clones isolated by Babinchak are available from NMFS

0.22µm polycarbonate membrane. This crude extract was first fractionated on a G-10 Sephadex size exclusion column (0.5m x 3.8cm i.d.), using gravity flow with a 100% methanol mobile-phase. The Sephadex CTX fraction, as determined by PbTx competition assay, was further fractionated on a C18 (30µm particle size) reverse phase silica column (1.5m x 3.8cm i.d.). The sequence of elution solvents was 100% water, 50%, 25%, 12% and 6% water:methanol using gravity flow.

IATROBEAD FRACTIONATION

A crude extract of 5g (wet weight) from each of 6 (MQ2, T04, G15, Hit25, G17 and T11B) mass cultured *G. toxicus* clones was prepared as

for the CTX standard. The methanol extract was evaporated to dryness and taken up to a final volume of 5-10ml chloroform. This solution was introduced to a Michel-Miller column (350mm x 40mm i.d.) equipped with a pre-column (130mm x 22mm i.d.). Both columns were packed with Iatrobeads, (porous, beaded silica; pH 6.8, 60µm particle size, 80Å pore size, Iatron Laboratories, Inc.). The solvent scheme used to fractionate the sample at pump flow rates of 5-8 ml/min was 100% chloroform, 2%, 5%, 10%, 15%, 25% and 50% methanol:chloroform, 100% methanol and finally 15% water:methanol.

MOUSE BIOASSAY

Column fractions were evaporated to dryness and brought up in methanol and dilutions made up in 0.9% saline containing 1% Tween 20. Toxicities were determined by injecting 0.25-0.5ml of appropriate toxin concentrations intraperitoneally into female ICR mice weighing c.20g. Three mice were initially injected per dose which was increased to at least 5 mice for doses used to determine the LD₅₀ toxicity. Total lethality is expressed in mouse units (MU), defined as the LD₅₀ dose for a 20g mouse over 48hr.

CELL CULTURE

Rat pituitary tumor cells (GH₄C₁) were maintained at 37°C and 5% CO₂ in Hams F10 nutrient mixture supplemented with 2.5% fetal bovine serum and 15% horse serum in the absence of antibiotics (Hams F10+). The cells were passed at weekly intervals to maintain their exponential growth, for a maximum of 10 passages. These cells were used in the cytotoxicity assay and calcium uptake experiments.

CYTOTOXICITY ASSAY

Cytotoxicity was determined by a modification of the procedure of Mosmann (1983). GH₄C₁ cells were plated using 0.1ml Hams F10+ in 96-well tissue culture plates at a concentration of 5.0 x 10⁵ cells/ml. Column fractions were evaporated to dryness and serially diluted in methanol. Duplicate wells of fraction-cell mixtures were then incubated for 18hr at 37°C. For determination of viability, 15µl of 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/ml in PBS) were added to each well and the cells incubated 4hr at 37°C. Mitochondrial dehydrogenases in live cells convert the MTT to an insoluble formazan crystal. After incubation, the cells were solubilized by addition of 10% SDS

in 0.1N HCl and absorbance at 570nm was recorded using a Titre Tek 96-well plate reader. Non-specific absorbance due to media and non-converted MTT were subtracted to yield a corrected absorbance value. Cytotoxicity was considered positive if the reading was within 10% of the positive control, negative if within 20% of the negative control and partial if in-between. In this study, a minimal lethal concentration (MLC) of toxin was defined as the last dilution to give only positive results.

CALCIUM UPTAKE EXPERIMENTS

Calcium uptake experiments were performed by a modification of the method of Enyeart et al. (1986). GH₄C₁ cells were plated in 96-well plates in 0.1ml of Hams F10+ and allowed to attach overnight. For the assay, the medium was replaced with Hams F10+ containing 5 μ Ci/ml ⁴⁵Ca²⁺ and test treatments. Cells were incubated with treatments for 10min at 37°C. To terminate the assay, the extracellular ⁴⁵Ca²⁺ was aspirated off and the cells rinsed 3 times with ice cold Hams F10+. Cells were solubilized by the addition of scintillation cocktail and the plates counted directly in a 96-well format microplate scintillation counter (Wallac). The calcium channel agonist, Bay K 8644, was used as a positive control. Samples were considered positive for values greater than 2 times the negative control.

BREVITOXIN (PbTx) DISPLACEMENT ASSAY

Assay of binding competition for the PbTx site on sodium channel receptors was carried out in 96-well plates by a modification of the method of Poli et al. (1986). All assays were carried out in the presence of binding buffer [50 mM Hepes, (pH 7.4), 130mM choline chloride, 5.5mM glucose, 0.8mM magnesium sulfate, 5.4mM potassium chloride, 1.0mg/ml BSA, and 0.01% Emulphor-EL-620]. To each sample well, a reaction mixture of 35 μ l of [³H]PbTx3 (5nM) and 35 μ l of test treatment were added in binding buffer. To this reaction mixture, 135 μ l rat brain synaptosomes in binding buffer were added. Plates were incubated at 4°C for 1hr, then receptor bound [³H]PbTx3 was trapped onto a glass fiber filter pad using a 96-well filtration apparatus (Millipore, Millipore). The filter pad was dried and impregnated with solid scintillant and bound [³H]PbTx3 detected by liquid scintillation spectroscopy in a 96-well format microplate scintillation counter (Wallac). Fractions which caused >50% decrease in [³H]PbTx3 binding were considered positive. PbTx-3(1 μ M) was

used as a positive control and caused complete inhibition of [³H]PbTx3 binding at the receptors.

RESULTS

At an inoculum level of 500 cells/ml, the cell yield of Martinique *G. toxicus* clone, MQ2, in mass culture (4532 \pm 526 cells/ml, N=44) was equivalent to the yield obtained in 1l of culture using 2.8l Fernbach flasks (4735 \pm 1105 cells/ml, n=26). Before an aeration system was introduced into the mass culturing system, cell yields with MQ2 (3325 \pm 502 cells/liter, n=52) were 30% less than produced in Fernbach culture. With aeration, the media pH rose to 8.8 units, but without aeration the rise was to pH 9.9. When the inoculum size for MQ2 was doubled, the final biomass yield increased 60% (0.85–1.3 g/l, wet weight).

Four clones that produce copious amounts of mucoid material in culture caused a problem in mass culture when aeration was introduced (Table 1). Bubbling created by the aeration drove the mucoid secretions and entrapped cells onto the wall of the flasks above the medium, separating the cells from their nutrients. When this occurred, mass culturing was terminated. Final yields in mass culture of the remaining 25 clones ranged from 0.6 to 1.5 g/l (wet weight).

While preparing the CTX standard, 2 suites of maitotoxin eluted within 4hr during fractionation on G-10 Sephadex. The CTX family of toxins eluted from the column between 10–48hrs, but peaked at 15hr. When the CTX fraction was run on the C18 column in the next stage of purification, fractions collected during elution with 100% water and 50%–25% water:methanol were weakly cytotoxic. These represent fractions where any remaining MTX not separated from CTX on G-10 would be expected to elute off the C18 column. No toxicity was observed again until CTX containing fractions were eluted with 12%–6% water:methanol.

The CTX fractions used as the CTX standard, 9.0ml total volume, had a mouse LD₅₀ toxicity of 0.007 μ l/MU for a total of 1.3 million MU produced from the 150g of MQ2 processed. The mice displayed intense lumbar contractions and progressive paralysis from hind to front legs. The toxin output for MQ2 was equivalent to one mouse unit per 2000 cells. The CTX standard had a cytotoxicity of 0.005 μ l/MLC and was negative or weakly positive in calcium flux and competitively inhibited labeled PbTx binding. The MTX fractions were calcium flux positive and did not inhibit PbTx receptor binding.

The toxin elution profiles for the 6 *G. toxicus* clones processed on Iatrobeads were similar and followed the solvent elution scheme. The first fraction isolated from the Iatrobead column with 100% chloroform was unique in that it displayed calcium flux activity, but was not toxic to mice or GH₄C₁ cells. CTX eluted in the next 1-3 fractions, 2%, 5% and occasionally 10% methanol:chloroform, inhibited PbTx binding, but did not affect mice or display cytotoxicity. After 1 or 2 fractions with no activity, 10% and 15% methanol:chloroform, MTX eluted with 25% and 50% methanol:chloroform and 15% water: methanol. The MTX fractions had high cytotoxicity, calcium flux activity, but did not inhibit PbTx binding on sodium channels receptors.

DISCUSSION

Dinoflagellates are generally considered sensitive to stirring in culture displaying cellular damage and reduced growth rates (White, 1976; Galleron, 1976; Tuttle & Loeblich, 1975). However, the agitation of the micro-carrier flask system maintained *G. toxicus* in suspension without any detrimental effect on its growth rate, and with aeration, the pH of the medium stabilized during the final growth phase and provided final yields equivalent to Fernbach culture. Silicates which dissolve off the walls and crystallize while autoclaving seawater in borosilicate bottles (Brand et al., 1981) provided fine attachment sites for the epiphytic *G. toxicus* in the medium. Aeration supplied the carbon needed for growth and also stabilized the pH in seawater by keeping it from rising too high through the interaction of the carbonate system with pH (Guillard & Keller, 1984). A pH-stat system (Goldman et al., 1982), would provide finer control of the pH as well as inorganic carbon for photosynthetic uptake. This should produce higher biomass yields and the reduction in creation of bubbles would also allow culturing of dense mucoid producing clones.

The initial mass culturing of MQ2 was a balance between using available incubator space for growing inoculum and mass culturing vessels. Because of this, inoculum size was limited to 500 cells/ml. By doubling the inoculum size of MQ2 in later studies, a 60% increase in the final yield was achieved which would be expected to occur with other clones if optimal inoculum were used. Limited culturing (n=3) using 12 litre polycarbonate culture vessels (Nalgene Corp.), with 18 litre working capacity, provided a 50% increase in culturing capacity with the same biomass

yield:medium volume ratio. The flasks occupied the same space as the borosilicate micro-carrier flasks, but were lighter and safer to handle.

It was critically important to filter and wash each inoculum and harvest cells in mid-Log growth phase while mass culturing. Neglect of these parameters increased the bacteria load and decreased growth vigor which resulted in reduced biomass harvest. Phenotypic changes that occurred during mass culturing that typified non-compliance to these parameters were increased mucoid production and adhesion of the cells to the vessel walls.

Wet weight yields at harvest were empirically related to the cellular volume of the clone. Generally, the larger the clone, the greater biomass produced per unit volume of medium. Acclimation to *in vitro* culture parameters was another determinate of biomass yield. Sixteen of the mass cultured clones were isolated less than 9 months previous to this study. *G. toxicus* can take up to one year to acclimate to culture conditions after isolation (Bomber et al., 1989). An increase in final yields would be expected for new isolates not yet acclimated to culture conditions.

The amount of CTX (purportedly) isolated from clone MQ2 using size exclusion G-10 Sephadex, followed by C18 reverse phase silica columns was 500-fold more on a per cell basis than previously isolated from this clone. However, previous separation of CTX from MTX was with a silicic acid column and a step-wise elution with chloroform and methanol (Tachibana, 1980), a separation technique recommended for use in ciguatera research (Anderson & Lobel, 1987). In the present study, the toxicity of CTX appeared to be unstable to contact with unprotected silica or the combination of silica and chloroform. This would explain the loss of toxicity, but retention of PbTx competitive binding activity when fractionated on Iatrobeads. To test this possibility, a sample of the CTX standard was fractionated on Iatrobeads. CTX was collected apart from the lipophilic, active calcium flux material using step-wise 100% ethyl acetate to 5% water/methanol eluents. The CTX standard lost 50% of its mouse toxicity and greater than 50% of its cytotoxicity. A hypothesis for this observation could be that CTX toxins exist in nature as epoxides. Though possibly still toxic after such an epoxide is opened, much of the toxicity would be lost on silica which may facilitate epoxide ring opening. Though only a hypothesis, the CTX structures (and MTX) that are known, would provide excellent sites for epoxide formation.

Since CTX toxins were not stable on an Iatrobeds packed column, this was not an ideal one-column procedure useful for rapid screening of *G. toxicus* clones for CTX production. However, they have proved to be excellent column packing for MTX purifications providing excellent separation with no loss in toxicity. Unlike other silica media, Iatrobeds allow higher concentrations of water to be used as eluant without destroying or dissolving the silica.

When separating CTX from MTX by normal phase chromatography, a non-polar, non-toxic, calcium flux active fraction, not previously identified, was included with CTX. This led us to believe that MTX was still present in these fractions. If hexane:methanol partitioning was used before column purification, this lipophilic compound was extracted in the hexane phase, unlike CTX or MTX.

Additional CTX extractions from clone MQ2 are in progress to determine if the high yield of CTX in this study was a function of the separation procedure or a serendipitous production of CTX by clone MQ2 in culture. Pacific *G. toxicus* clone, G15, which produces several interesting fractions (isolated from the Iatrobed column) that competitively displaced PbTx binding to sodium channels, is now in mass culture. Although the identity of the CTX standard was confirmed with a negative calcium flux assay and positive competition for [³H]PbTx3 binding, its yield from cultured *G. toxicus* is 500 times that previously reported (Balthrop & Herring, 1990), which leaves lingering doubts that the CTX standard contained only CTX congeners. To eliminate this uncertainty, further production and purification of the CTX standard using HPLC and definitive confirmation of the compounds with mass spectroscopy and NMR are in progress.

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